Saskatchewan Agriculture, Food and Rural Revitalization

ADF

AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

19970272

INSECT AND DISEASE RESISTANCE IN CANOLA

Funded by: The Agriculture Development Fund

February 2003

Prepared by: Agriculture and Agri-Food Canada

106-01272/

Final Report to ADF

Date: November 04 2002

Reporting Period: June 1998 – August 31 2002.

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Project: Insect and Disease Resistance in Canola

Abstract:

The objective of the project was to develop strategies that can be used to generate canola germplasm that is resistant to attack from insects and disease. Our strategy involved the engineering of highly specific inhibitors of enzymes, namely proteases, which are used by these organisms to digest protein in plant tissues. We have discovered that insect pests of canola use two major classes of digestive proteases, the serine and cysteine proteases. We have developed and continue to develop extensive libraries of protease inhibitor variants from which specific inhibitors that are highly effective against a given pest can be selected. The net result is the generation of a plant on which the pest is unable to survive.

Executive summary:

The project began in mid-1998 and we have met the milestones outlined in the original proposal and detailed below. Collaboration between AAFC-Saskatoon and AAFC-Ottawa allowed us to meet these commitments and to explore new opportunities for protease inhibitor design and insect resistance.

Overall, we have identified the major digestive proteolytic enzymes used by the following canola pests; flea beetle, root maggot, bertha armyworm, diamondback moth and *Lygus* bug. Genes encoding 44 proteases (8 from root maggot, 35 from Bertha armyworm and 4 from the diamondback moth) were isolated. AAFC-SRC provided additional support for this work and has committed to sequence up to 10,000 canola pest expressed sequence tags (ESTs) primarily from the midgut-specific cDNA libraries developed through this project. To date, 1,092 ESTs from the bertha armyworm have been sequenced revealing the genes for the possibly the entire complement of digestive proteases.

We have developed three phage display libraries of protease inhibitory proteins effective against general cysteine and two classes of serine proteases. We have demonstrated that unique inhibitors can be selected from the libraries using the flea beetle digestive protease as the target. We have discovered a novel class of protease inhibitors present within the bertha armyworm midgut that appear to be involved in a unique mechanism to regulate digestive enzyme synthesis and activity. We have expressed three of these protease inhibitor genes and purified sufficient amounts to examine their bioactive properties. Collaborators in Ottawa have generated lines of *B. napus* expressing functional serine and cysteine protease inhibitors. A full US patent was

filed in December 2000 whereby expression of a cysteine protease inhibitor in *B. napus* constitutes one of the major embodiments. A second invention disclosure has been submitted describing three novel protease inhibitors discovered in the bertha armyworm.

The bulk of our research activities in the final stages of the project have been devoted to the selection of specific protease inhibitors active against flea beetle proteases. We have now completed the characterization of the digestive biochemistry of this pest and have isolated a series of novel engineered cysteine protease inhibitors that interact with flea beetle digestive proteases. These interactions are now being confirmed biochemically and the proteins are being expressed and purified to provide material for bioassays.

At present, three publications, one patent, one invention disclosure, three book chapters/review articles and eight conference presentations, one CBC radio interview describing aspects of this work have been released. The residual information will contribute to additional publications.

The materials developed through this project are currently being made ready for larger scale bioassays. The investigators are considering new project applications to ADF within the next year pertaining to the further development of insect-specific targeting strategies and the manipulation of natural plant defenses.

Technical Report

Phase I (Protease Profiles and Gene Isolation, Year 1-2)

Milestones (1998-99)

1) Identification of Digestive Proteases

The work, as outlined in the proposal, was completed and this aspect of the research was extended to provide a thorough analysis of the digestive processes for select canola pests. A new methodology to resolve protease isoforms using multi-dimension electrophoresis was adapted for the identification of canola pest digestive proteases. We found that two Lepidopteran pests, the diamond back moth and the bertha armyworm (BAW), possess exclusively the serine class of proteases in their digestive tracts. The proteases from bertha armyworm have been extensively characterized and fall into three major serine protease subclasses, the trypsins, elastases and chymotrypsins, representing approximately 90 % of total protease activity, respectively. We estimated that at least 15-20 protease isoforms are responsible for digestion of proteins by BAW and we were able to perform fine separations of these digestive proteases. Despite the number and diversity of serine proteases present in bertha armyworm, we demonstrated that this insect is sensitive to incorporation of relatively small amounts (0.2%) of soybean trypsin inhibitor in the diet formulation. This level is achievable in modified plants.

As stated in the original proposal, we previously generated a canola line expressing a serine protease inhibitor that greatly reduced the hatching of *Lygus* bug fecundity, ie. egg laying, egg hatching and subsequent development of the larvae. The root maggot was found to possess both serine and cysteine protease activity, although we discovered that two cysteine protease activities are involved in insect development rather than digestion. If sufficient resources become available, we wish to continue investigating whether targeting of developmental proteases is an effective strategy to control insect populations.

We have demonstrated that the major digestive protease activity in adult flea beetle is provided by 4-6 cysteine proteases. We have now fully characterized the digestive protease activity of the flea beetle using a battery of highly specific substrates and biochemical tests. We also have evidence to suggest that a single aspartic protease, in addition to the cysteine protease previously identified, also contributes to the hydrolysis of plant proteins.

2) Construction of midgut cDNA Libraries

The work, as outlined in the proposal, was completed and this aspect of the research was applied to the isolation of insect digestive protease genes as described below.

cDNA libraries representing the genes expressed in the midgut of bertha armyworm, diamondback moth and root maggot were constructed. A cDNA library representing whole flea beetle was also prepared, however, we now isolated sufficient flea beetle midgut tissues and developed a protocol to construct a midgut-specific cDNA library for this insect. AAFC-SRC has provided additional support to sequence up to 10,000 canola pest expressed sequence tags (ESTs) from the midgut-specific cDNA libraries developed through this project. To date, 1092 ESTs from the bertha armyworm have been sequenced revealing the genes for the entire complement of digestive protease, many other digestive enzymes and has provided new prospects for insect control.

Phase II (Selection of Novel Protease Inhibitors, Years 2-3)

Milestones (1999-2000)

1) Completion of Protease Inhibitor Libraries

The work, as outlined in the proposal, was completed and applied to the selection of highly specific protease inhibitors against flea beetle proteases.

Three phage display libraries based on the *Onchocerca volvulus* cysteine protease inhibitor (OV7) gene were completed; containing 3,000, 300,000 and 3,000,000 variants, respectively. Two serine protease libraries based on the dual function soybean Bowman-Birk inhibitor were completed. One library encoded variants of the trypsin inhibitor region and the other the chymotrypsin inhibitor portion. Both the wild-type trypsin and chymotrypsin inhibitors were expressed separately on phage and found to be fully functional. Colleagues in Brazil panned these Bowman-Birk libraries against proteases from a sugarcane pest and isolated unique variants. We screened the OV7 libraries against flea beetle protease preparations (see below).

As reported previously, in screening the BAW midgut cDNA library for additional protease genes we discovered the genes for three unique serine protease inhibitors. The presence of such proteins in the insect midgut was a very novel discovery. We expressed the genes for three of these novel inhibitors in bacteria, yeast and baculovirus and were able to purify sufficient amounts of these proteins to test insect bioactive potential. We have also identified the regions of these inhibitory proteins responsible for the specific interaction with target proteases and are focusing upon this region for further inhibitor improvement.

2) Isolation of Insect Protease Genes

Using a Genomics approach eight protease genes have been isolated from a root maggot midgut cDNA library, one encoding a cysteine protease that is important for insect development and 7 serine proteases that are involved in digestion. Another 35 serine protease genes have been isolated from Bertha armyworm, comprising three major enzymatic groups (trypsins, chymotrypsins and elastases). Due to the limited number of flea beetle digestive proteases it was not necessary to isolate and express individual genes. We have also initiated on a genomics project to sequence the entire complement of genes expressed in the flea beetle midgut. In addition to identifying genes encoding digestive proteases other unique targets may be identified. Genes encoding 4 digestive proteases were isolated from the diamond back moth.

Recently, in collaboration with A. Ross (NRCC-PBI) and D. Smith (University of Victoria) we have introduced new techniques that allow for rapid identification of genes encoding target proteins. Briefly, two-dimensional electrophoresis is used to separate proteins followed by mass spectrographic analysis and protein sequence determination. This data is being used to identify the genes encoding these proteins from the insect midgut EST database.

Milestones (2000-01)

a) Selection of Protease Inhibitors from Cysteine and Serine Libraries

We have completed the screening of two OV7 cysteine protease inhibitor libraries for inhibitors active against flea beetle protease extracts. Protocols to carry out these experiments were originally established using the papaya cysteine protease, papain, and several novel

inhibitors selected. We are in the process of testing these inhibitors for specificity, biochemical activity and insect bioactivity.

b) Assessment of Insect-Inhibitory Activity in Laboratory Feeding Bioassays

We developed an artificial diet for the BAW with a greatly reduced protein content. This formulation better reflects natural conditions and we demonstrated that plant protease inhibitors (specifically the soybean trypsin inhibitor) have an adverse effect on BAW development. We tested one serine protease inhibitor derived from the BAW itself in feeding bioassays to determine their effect on larval weight gain, mortality and digestive protease regulation. Only a small amount of the protein was available for bioassay. The initial results with a small number of insects indicated the protein had some effect, however, these findings require confirmation. During the summer of 2002, a summer student was employed to refine procedures for large scale protein purification which will allow us to isolate sufficient material for further bioassays.

c) Assessment of Inhibitory Activity Against canola Pathogens

The project proposal stated that canola fungal pathogens, such as *Leptosphaeria maculans*, *Sclerotinia sclerotiorum*, *Alternaria brassicae* and *Rhizoctonia solani*, might also use proteases to degrade plant tissues. We showed that *S. sclerotiorum* secretes a small amount of serine protease. The significance of this activity to the infection process remains to be determined. During the summer of 2002, the summer student developed assays to examine the growth inhibitory effects of exogenous peptides and proteins with the four pathogens noted above.

Phase III (Generation of Transgenic Canola and Gene Pyramiding, Years 3-4)

Milestones (2001-02)

Originally, we used the CaMV 35S promoter to drive the expression of the OV7 cysteine protease inhibitor gene in *B. napus*, however, this did not result in high levels of gene expression. To improve the levels of expression we used a proprietary AAFC promoter, HPL, developed at the Saskatoon Research Centre.

We now have several *B. napus* lines transformed with HPL -OV7, 70S-OV7, and Bn115-OV7 (cold-inducible). The presence of the OV7 gene has been confirmed using PCR and Northern blot analysis show proper constitutive expression under the HPL and 70S promoters and cold-induction of the gene under the direction of Bn115. The Bn115 should direct expression under the environmental conditions associated with cotyledon emergence and when the plants are most susceptible to flea beetle attack. These lines are currently undergoing analysis to determine protein expression levels and homozygous lines are being selected prior to seed increase for greenhouse and field bioassays.

A full US patent was filed in December 2000 and expression of a cysteine protease inhibitor in *B. napus* constituted one of the main embodiments.

Personnel:

Name	Position/Level	Source	Time
Mr. Doug Baldwin	Technician	ADF	75%
		CCC	25%
Ms. Kalindi Dill	Summer Student	ADF	100%

Project Developed Materials:

Two randomly mutagenized cysteine protease inhibitor libraries.
Two randomly mutagenized serine protease inhibitor libraries.
Three midgut-specific cDNA libraries from canola pests.
1092 ESTs from BAW midgut.
Three novel protease inhibitor genes from BAW.
Genes encoding 44 canola pest digestive proteases.
B. napus lines expressing the OV-7 cysteine protease inhibitor gene.
Cysteine protease inhibitor variants selected against flea beetle proteases.

Project Photos: None at this time.

Acknowledgements: ADF support of the project was acknowledged in the following:

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Patents and Disclosures

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Media Interactions

Interview with Marshall Perrin, AAFC correspondent with CBC radio, regarding biotechnological approaches to control of insect pests. Aired during the summer of 2002.

Expense Statement: Attached

Dwayne D. Hegedus
Research Scientist

cc: M. Erlandson (AAFC-SRC)
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